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(54) **Process for the preparation of aqueous formulations for ophthalmic use**

(57) The present invention describes a process for the preparation of aqueous formulations containing azithromycin for ophthalmic use, as well any formulations

obtained by this process and their topical use in the ocular bacterial infections, more preferably in the treatment of conjunctivitis, keratitis and blepharitis.

**EP 1 075 837 A2**

## Description

**[0001]** The present invention refers to a process for the preparation of aqueous formulations for ophthalmic use. Specifically, the present invention relates to a process for preparing ophthalmic formulations containing azithromycin, as well any formulations obtained through a such process, and their ophthalmic use against ocular bacterial infections caused by gram-positive and gram-negative pathogens (e.g. *Staphylococcus spp.*, *Streptococcus spp.*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter*, *Citrobacter*, *Chlamydia spp.*) as well as from other microorganisms generally involved in the most common ocular infections (e.g. conjunctivitis, keratitis and blepharitis).

**[0002]** Azithromycin (US 4517359) is a well-known antibiotic belonging to the macrolide class (of which erythromycin is the precursor), antibiotics having a structural similarity, most of them isolated from fermentation of *Streptomyces spp.*, and essentially utilized in the treatment of the skin and soft tissue infections caused by gram-positive organisms, even though the spectrum of action of the newer macrolides also includes some gram-negative organisms (e.g. *Haemophilus influenzae*). Notwithstanding the structural similarity, azithromycin can be considered as unique within the macrolides class, such as to be included in a new class of antibiotics known as azalides. In particular, the specific characteristics of azithromycin make this molecule more stable, tolerated and effective than its precursor erythromycin (S. Alvarez-Elcoro, M. J. Enzler, "The macrolides: Erythromycin, clarithromycin, and azithromycin", Mayo Clinic Proceedings, 1999, 74: 613-634).

**[0003]** In fact, erythromycin and its salt derivatives (e.g. erythromycin lactobionate, erythromycin glucoheptonate, erythromycin estolate, erythromycin succinate etc.) have often been shown unstable in acidic medium and physiological conditions as well, by causing degradation products in microbiologically-inactive structures [P. J. Atkins et al., "Kinetic studies on the decomposition of erythromycin A in aqueous acidic and neutral buffers", Int. J. Pharmaceutics, 1986, 30: 199-207; E. Fieser, S. H. Steffen "Comparison of the acid stability of azithromycin and erythromycin A", J. Antimicrob. Chemother., 1990, (Suppl. A) 25: 39-47; M. M. Amer, K. F. Takla "Studies on the stability of some pharmaceutical formulations. V-stability of erythromycin", Bulletin of the Faculty of Pharmacy Cairo University].

**[0004]** In addition azithromycin, even in comparison to other recent macrolides, shows a superior antibacterial activity against some gram-negative organisms, while retaining the same efficacy against gram-positive organisms, moreover azithromycin, with respect to other macrolides, has an extensive intracellular distribution into specific tissues after oral administration (R. P. Glaude et al., Antimicrob. Agents and Chemother., 1989, 33(3): 277-82). Half-life of azithromycin is so extremely elevated such as to be considered an excellent antibiotic, after a once-daily administration, against infections of the respiratory tract, skin and soft tissues [A. P. Ball et al., J. Int. Med. Res., 1991, 19(6): 446-50; A. E. Girard et al., Antimicrob. Agents and Chemother., 1987, 31(12): 1948-1954].

**[0005]** Furthermore, it is also possible to administer azithromycin, by systemic route, in a variety of preparations and pharmaceutical forms. However, even though the characteristics of this molecule are such as to privilege its use as antibacterial in the topical ocular administration as well, so far it has been failed to prepare aqueous formulations for ophthalmic use, containing azithromycin, stable and compatible to the ocular structures.

**[0006]** Among the major difficulties to overcome, in providing an aqueous ophthalmic preparation of azithromycin, is the poor water solubility of this molecule together with safety problems resulting from the potential ophthalmic use of one of its salts, obtained by applying classical criteria of chemical synthesis, wherein the purification of the organic solvents being utilized, harmful to the ocular structures, is extremely difficult and often not completely resolvable.

**[0007]** As an example, EP-B-0677530 and US 4474768 patents describe the preparation of different azithromycin salt derivatives, in presence of organic solvents, pharmaceutically acceptable, wherein before utilizing them in therapy, through pharmaceutical forms usually administered as oral or other incompatible forms with the topical ophthalmic use, their purification methods have to be repeated many times. It has also been described how is unlikely to overcome the difficulties of pharmaceutical type, essentially because of the poor aqueous solubility of macrolides (V. Andrews, "Antibiotic treatment of ophthalmic infection: new developments", J. Hospital Infection, 1995, 30: 268-274), and, although their acquisition in ophthalmic therapy has been wished, unless to rely on ophthalmic forms (e.g. ointment) less bioavailable and which, anyway, it would make necessary their combination with eye drops of the same active principle, this in order to completely eradicate after treatment any pathogen distributed into the ocular surface.

**[0008]** Numerous publications describe the pharmacokinetics of azithromycin after oral administration together with its potential application to treat infections of the ocular structures [K. F. Tabbara et al., "Ocular levels of azithromycin", Arch Ophthalmol., 1998, 116(12): 1625-1628; Z. A. Karcioğlu et al., "Pharmacokinetics of azithromycin in rabbit lachrymal glands and conjunctiva", Ophthalmic. Res., 1999, 31(1): 47-52; K. F. Tabbara et al., "Single-dose azithromycin in the treatment of trachoma. A randomized controlled study" Ophthalmology, 1996, 103(5): 842-846; D. M. O'Day et al., "Ocular pharmacokinetics of orally administered azithromycin in rabbits", J. Ocular Pharmacol. 1994, 10(4): 633-641; Z. A. Karcioğlu et al., "Pharmacokinetics of azithromycin in trachoma patients: serum and tear levels", Ophthalmology, 1998, 105(4): 658-661]; however, under no circumstances the oral formulations utilized are able to make sure an effective tissue concentration of azithromycin into the ocular surface, what it would occur by administering

topically the active principle in the elective pharmaceutical form of eye drops.

[0009] Whether from one side the synthesis of azithromycin salt derivatives has been improved or in some other instances it has been tried to increase the bioavailability as well as the activity through oral administration by adopting controlled release systems, see US patent n. 5705190 referred to clarithromycin, from the other hand it has not been able to obtain similar results in preparing stable aqueous formulations, containing azithromycin, to be utilized as effective and safe products in the antimicrobial therapy of ocular infections.

[0010] It is an object of the present invention to have stable aqueous formulations for ophthalmic use containing azithromycin, providing a better corneal permeability of the active ingredient, with respect to the aqueous suspensions or lipophilic formulations, with a superior bioavailability and compatibility to the ocular structures.

[0011] It is another object of the present invention to prepare formulations whose process of preparation does not include the presence of organic solvents to be utilized either as cosolvents during the preparation of azithromycin eye drops, or as precipitating agents during the process of purification in preparing azithromycin salt derivatives by synthesis.

[0012] Moreover it is desirable to be able in realizing aqueous ophthalmic formulations containing azithromycin, which are exploitable as eye drops in the antimicrobial ophthalmic therapy.

[0013] The objects described above and other of the present invention, which will become better understood from the following description, have been surprisingly achieved through a process for the preparation of an aqueous ophthalmic formulation containing azithromycin which comprises the solubilization of ophthalmically acceptable polybasic phosphate in a concentration range from 7.8 to 68.6 g/l, citric acid monohydrate in an amount ranging from 0.9 to 35.94 g/l, and the subsequent addition of azithromycin in an amount ranging from 0.1 to 100 g/l, within a temperature range from 15 to 25 °C, wherein the molar ratio of azithromycin to citric acid is about 1:0.67 to 1:1.5; wherein pH is adjusted to a value of 5.5-7.6, and up to a final osmolality between about 130 to about 300 mOsm/Kg.

[0014] In one preferred embodiment of the present invention an ophthalmically acceptable polybasic phosphate is sodium phosphate, more preferably disodium phosphate dodecahydrate. The solution, under the process of the present invention, has preferably a pH ranging from about 6.4 to about 7.6, wherein the most preferred molar ratio of azithromycin to citric acid is equal to 1.5:1.

[0015] The process of the present invention allows to obtain an extremely high solubility of azithromycin in aqueous solution, also superior than 10% w/v. Formulations containing azithromycin at concentration ranging from 0.01 to 10% w/v, more particularly between 0.3 and 5% w/v, are the most preferred.

[0016] According to another preferred aspect, the process of the present invention, comprises, subsequently to the azithromycin dissolution, the addition of at least a tonicity agent and/or a viscosity-increasing agent and/or a gelling agent and/or a stabilizing agent and a preservative agent, in amounts ophthalmically acceptable.

[0017] Aqueous ophthalmic formulations obtained as defined in the process of the present invention are novel and, according to another preferred aspect, comprise, in combination with azithromycin, at least another therapeutic agent having antibacterial activity and/or a therapeutic steroidal or nonsteroidal agent having antiinflammatory activity in amounts ophthalmically acceptable to the eye.

[0018] In particular, the therapeutic agent having antibacterial activity is selected from the group consisting of aminoglycosides (e.g. netilmicin), fluoroquinolones, tetracyclines, polymyxin, glycopeptides, glycoproteins (e.g. lactoferrin), natural and/or synthetic peptides,  $\beta$ -lactam antibiotics, as well as other antibacterial agents, whereas the therapeutic steroidal agent having antiinflammatory activity is selected from the group made up of desonide 21-phosphate, dexamethasone, clobetasone, mometasone, betamethasone, fluticasone and other similar steroidal antiinflammatory agents. Non-steroidal agent having antiinflammatory activity is selected from the group made up of naproxen, diclofenac, nimesulide, flurbiprofen and other similar non-steroidal antiinflammatory agents.

[0019] It is a preferred object to realize the formulations of the present invention as aqueous solutions, ointment or gel forms as well as other systems of release ophthalmically compatible to the ocular structures.

[0020] Formulations of the present invention can be advantageously utilized for the preparation of a medicine in the treatment of ocular pathologies requiring antibacterial therapy, more preferably in the treatment of conjunctivitis, keratitis and blepharitis.

[0021] FIG.1 shows the titration curves of azithromycin, dispersed in water, with organic acids (citric acid and tartaric acid) and inorganic acids (phosphoric acid, hydrochloric acid); x-axis represents the acid concentration ( $\mu$ M), whereas the y-axis represents the corresponding pH. Although any addition of acid improves the solubility of the azithromycin (sigmoidal curve), citric acid allows of obtaining instantaneously an elevated concentration of azithromycin during the process for the preparation of eye drops, within a physiological range of pH.

[0022] FIG. 2 shows titration curves, similar to that illustrated in FIG.1, in which azithromycin and clarithromycin are titrated with citric acid; the equivalent point for clarithromycin is about pH 4.5 instead of about pH 7.0 for azithromycin. Thus, the chemical interaction between citric acid and clarithromycin does not occur at physiological pH. In addition, the maximum concentration of clarithromycin in water (< 2%) is markedly lower than that achievable for azithromycin.

[0023] FIG.3 shows the pH-rate stability profile of a solution containing 2% of azithromycin under thermal stress

condition. Log K (week<sup>-1</sup>) is plotted (y-axis) against pH (x-axis). Formulations of the invention are stable at pH 6.4 and pH 8.7.

[0024] FIG. 4 shows the ocular distribution of azithromycin into the cornea and conjunctiva (T/g of tissue, y-axis) as a function of the time (hours, x-axis), after topical treatment of the animals. Tissue concentrations of azithromycin after twelve hours of treatment is approximately double with respect the initial three instillations, and these values are maintained above MIC<sub>90</sub> for *Staphylococcus aureus* also in the group of animals left untreated for twelve hours.

FIG. 5 shows the bacterial burden reduction in three groups of rabbits, having ocular bacterial conjunctivitis, treated with different solutions of azithromycin eye drops prepared under a variety of concentrations (x-axis), as defined in the process of the present invention, in which colony-forming units (cfu), expressed as log cfu/g of tissue (y-axis), are determined. In accordance with the activity profile of each tested concentration, it appears evident how the eye drops of the present invention is appropriate for the topical treatment of the ocular bacterial conjunctivitis.

[0025] FIG. 6 shows the bacterial burden reduction in three groups of rabbits, having ocular bacterial keratitis, treated with different solutions of azithromycin eye drops prepared under a variety of concentrations (x-axis), as defined in the present invention, in which colony-forming units (cfu), expressed as log cfu/g of tissue (y-axis), are determined.

[0026] Based on the lower mean observed for the reduction of the bacterial burden into the corneas treated with azithromycin (2%), with respect to the vehicle, it is possible to sustain that higher concentrations of azithromycin (>2%), administered as ointment or gel pharmaceutical forms and other ocular release systems as well, are effective in the ocular therapy of bacterial keratitis.

[0027] The process of the present invention is able to overcome any difficulties in preparing aqueous compositions containing azithromycin. In fact, it has been discovered a process in which, without being necessary to synthesize azithromycin salts in presence of organic solvents, it is possible, by adding appropriate amounts of citric acid/phosphate buffer ratio to the azithromycin suspension, to obtain a stable aqueous pharmaceutical form which is compatible to the ocular structures. Aqueous solutions of azithromycin prepared as defined in the process of the present patent, wherein pH ranges from 5.5 to 7.6 and osmolality ranges from 130 to 300 mOsm/Kg, are able to achieve tissue concentrations above the MIC values of the most common ocular pathogens (e.g. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus viridans*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae*, *Enterobacter*, *Citrobacter*, *Haemophilus influenzae*, *Chlamydia spp.*) making them effective in the treatment of the major ocular bacterial infections.

[0028] Ophthalmic formulations prepared as defined in the process of the present invention can comprise, in an amount ophthalmically acceptable, a polybasic phosphate buffer; viscosity-increasing agents and gelling agents (e.g. salts of hyaluronic acid, ophthalmically acceptable, with a molecular weight range of from about 200.000 to about 5.000.000 Dalton, Lutrol<sup>(R)</sup> F127 (BASF), Kollidon<sup>(R)</sup> (BASF), hydroxypropyl methylcellulose, Carbopol<sup>(R)</sup> (Goodrich), stabilizing agents (e.g., polyethylene glycol, propylene glycol, Cremophor<sup>(R)</sup> (BASF), polysorbate, ascorbate, ionic surfactants) preservatives (e.g. benzalkonium chloride, cetrimide, thimerosal, chlorobutanol, p-hydroxybenzoate, polyhexamethylen biguanide, clorexidine, sorbates), tonicity agents (sodium chloride and/or potassium chloride, glycerol, mannitol) and/or other excipients (e.g., vaseline, paraffin oil, lanolin, etc.) commonly utilized in ophthalmic formulations for human and veterinary use.

[0029] The surprising effect of the citric acid in solubilizing azithromycin, together with the unexpected opportunity to obtain elevated concentration of azalide in physiological pH range, have been exploited for preparing various azithromycin eye drops at different concentrations and pH range, in order to evaluate their stability, pharmacokinetics, safety and efficacy.

[0030] The following examples are for illustrative purposes only and are not to be interpreted as limiting the scope of the invention.

#### EXAMPLE 1

##### Preparation of azithromycin eye drops

[0031] In 80 ml of water have been added, while stirring at temperature of 15-25°C, respectively as follows, disodium hydrogen phosphate dodecahydrate and citric acid monohydrate, azithromycin and benzalkonium chloride. Azithromycin has been added only when buffer agents are completely dissolved. After the complete dissolution of all the ingredients, including suitable tonicity agents, viscosity-increasing or gelling agents, stabilizing agents and additional therapeutics agents (e.g. antibiotics, nonsteroidal or steroidal antiinflammatory drugs) pH has been measured and afterward, if necessary, this value has been adjusted to pH=6.4-7.6 with 1M citric acid or 1M NaOH. The final solution (100 ml), has been sterilized and distributed in appropriate vials. Some formulations derived from this procedure are shown in the table below:

	Azithromycin (%;w:v)	Na <sub>2</sub> HPO <sub>4</sub> 12H <sub>2</sub> O (%;w:v)	Citric acid (% w:v)	
	Other active(%;w:v)			
5	0.3	3.300	0.223	BK
	1	3.300	0.440	BK
	2.0	3.300	0.536	BK
10	2.0	3.300	0.536	BK, NaHA
	2.0	3.300	0.370	BK, NaCl
	2.0	3.300	0.370	GL, BK
	5.0	2.54	1.022	BK
15	2.0	3.300	0.440	TH
	Naproxen sodium salt (0.2)			
	2.0	3.300	0.440	TH
20	Diclofenac sodium (0.1)			
	2.0	3.300	0.370	BK, NaCl
	Netilmicin sulfate (0.455)			
	2.0	3.300	0.440	BK
25	Lactoferrin (2.0)			
	2.0	3.300	0.440	BK
	Dexamethasone sodium phosphate (0.2)			
30	2.0	3.300	0.440	BK, NaHA, PS
	Mometasone furoate (0.2)			
	1.0 (ointment)	0.780	0.229	PO, VA, LA
	1.0 (ointment)	0.780	0.229	PO, VA, LA
35	Mometasone furoate (0.2)			
	2.0	3.300	0.536	BK, LU
40	GL = Glycerol: 1.5% (w:v) BK = Benzalkonium chloride: 0.005% (w:v) TH = Thimerosal: 0.005% (w:v) LU = Lutrol F127: 15.5% (w:v) NaHA = Sodium Hyaluronate: 0.15% (w:v) PS = Polysorbate 80: 0.2% (w:v) NaCl = Sodium Chloride: 0.800 (w:v) PO = Paraffin oil: 20% (w:w) VA = Vaseline: up to 100g (w:w) LA = Lanolin : 10+10% H <sub>2</sub> O			

50 **EXAMPLE 2****Stability test**

55 **[0032]** Under thermal stress conditions (60°C ± 2°C) aqueous formulations show the best stability at pH=6.4 and pH=8.7 (see FIG.3). Azithromycin formulations (EXAMPLE 1) at physiological pH (6.4-7.6) and at temperature of 25°C ± 2°C; with 75% ± 5% of relative humidity, are still stable after 4 weeks from their initial preparation as shown in the following table.

Formulation	Weeks	pH	Azithromycin remaining (%)
pH 5.73	0	5.73	100.0
	1	5.60	98.8
	4	5.64	96.2
pH 6.42	0	6.42	100.0
	1	6.27	99.6
	4	6.30	99.8
pH 7.10	0	7.10	100.0
	1	6.97	100.1
	4	6.96	100.2
pH 7.66	0	7.66	100.0
	1	7.52	99.5
	4	7.47	100.2
pH 8.03	0	8.03	100.0
	1	7.41	96.7
	4	7.38	89.6
pH 8.68	0	8.68	100.0
	1	8.05	94.1
	4	8.00	84.0

**[0033]** Azithromycin concentration does not affect the stability of the final product; all the solutions, even after 4 weeks, are clear and colorless as well.

### EXAMPLE 3

#### Ocular tolerability

**[0034]** Twenty-four albino New Zealand rabbits (12 males and 12 females), weighing 1.9-2.0 kg, have been randomly distributed in groups (n = 2) for being topically treated with azithromycin (2%) eye drops or placebo in according to the following scheme:

Group	Treatment		Animal number	
	Right eye	Left eye	Male	Female
1	Eye drops	Untreated	13,14,15, 16,17,18	1,2,3,4, 5,6
2	Placebo	Untreated	19,20,21, 22,23,24	7,8,9,10, 11,12

**[0035]** Eight treatments the first day and four the following days have been performed. A drop (50 µl) of azithromycin (2%) eye drops or placebo has been administered into the conjunctival *cul-de-sac* of the animal. After the administration the lid has been maintained close for a couple of seconds in order to reduce loss of solution and to permit its distribution into the eye. Differences between each treated group and their corresponding control have been assessed by Mann-Whitney test.

**[0036]** By using a slit-lamp, clinical examination of the eyes has been performed at 0, 1¼, 1½, 1, 2, 3, 4, 5, 6, 7, 8, 24, 48, 72, 96, 168, 240, 360, 480 and 672 hours after the beginning of the study. Ocular tolerability/toxicity has been established by recording the clinical signs in the conjunctiva (congestion, edema, exudate), cornea (opacity) and iris (dilation) according to the scoring system developed by Draize. The degree of severity of each sign has been graded from 0-3 (0=normal, 1=mild, 2=moderate, 3=severe).

**[0037]** After clinical observation, four corneas for each group of rabbits have been processed for scanning electron microscopy (SEM). In addition, after treatment various organs and tissues have been stored away for the histopathologic examination.

**[0038]** No clinical signs indicating any effect related to the azithromycin (2%) eye drops has been observed during the 28-days treatment. The Draize test has not shown significant difference between azithromycin (2%) eye drops and

placebo.

[0039] Analysis by SEM and histopathologic examination as well showed no treatment-related change in the tissues and organs examined (eye, heart, brain, liver, kidney, lung, colon and stomach). As a result azithromycin (2%) eye drops appears to be well tolerated following topical instillation into the eye of albino rabbits during a 28-days period.

#### EXAMPLE 4

##### Azithromycin distribution in conjunctiva, cornea, and aqueous humor after topical application in the eye of albino rabbits

[0040] A pharmacokinetic study it has been carried out in order to estimate the concentration of azithromycin in the cornea, conjunctiva and aqueous humor. Twelve male white New Zealand rabbits (weighing 1.8-2.3 kg) have been randomly divided in three groups (consisting of four animals each). Subsequently, all rabbits have been instilled in the lower conjunctival *cul-de-sac* of both eyes, every two hours, with 50  $\mu$ l of azithromycin (2%) according to the following dosing scheme:

- I Group : three instillations  
(animals have been sacrificed 6 hours after the first dosing)
- II Group: six instillations  
(animals have been sacrificed 12 hours after the first dosing)
- III Group: six instillations  
(animals have been sacrificed 24 hours after the first dosing)

[0041] Rabbits have been sacrificed by an overdose of Tanax<sup>R</sup> injected into the marginal ear vein, the eyes treated with azithromycin have been washed with phosphate-buffered saline (PBS 0.1 M; pH 7) and each conjunctiva has been surgically removed just before enucleation. Aqueous humor has been drawn away with a 23-gauge needle attached to an insulin syringe of 1 ml. After enucleation the cornea has been excised at the limbus and removed from the eyeball. All specimens have been carefully weighed, then immediately frozen at -20°C.

[0042] Tissue or aqueous humor concentrations of azithromycin have been determined by the following microbiologic method. Each specimen has been ground and homogenized with 1 ml of PBS (2 min., 24,000 rpm). The suspension has been mixed with an equal volume of acetonitrile and stored at 0°C. After 1 hour, the samples have been centrifuged at 0°C (12,000 rpm; 10 min) and the aqueous supernatant layer has been evaluated for the azithromycin measurement in each specimen. Aqueous humor has been directly mixed with acetonitrile before being centrifuged. Azithromycin concentrations for each cornea, conjunctiva and aqueous humor have been determined in triplicate, averaged for each tissue sample, and compared to a standard curve. It has been confirmed a linear relationship between azithromycin concentration (log) and its related inhibition zone against *Staphylococcus aureus* ATCC 29213 as shown from the following regression lines determined for each sample:

Sample	Regression line
Cornea	$Y=6.3312 x + 7.5975$ ; $r=0.9980$
Conjunctiva	$Y=6.2464 x + 7.3909$ ; $r=0.9999$
Aqueous humor	$Y=9.2474 x + 4.6451$ ; $r=0.9864$

[0043] The bioassay method has a detection limit of 3.8 Tg/ml in the aqueous humor.

[0044] Azithromycin concentration increased in both cornea and conjunctiva during the first twelve hours (see tables below and Fig. 4). In particular, tissue concentration of azithromycin after 12 hours (six instillations) is about twice as much that evaluated after six hours of treatment. Finally, even after 24 hours and 6 instillations the concentration of azithromycin in both cornea and conjunctiva is still maintained above MIC<sub>90</sub> for *Staphylococcus aureus* (2.25 Tg/ml). These data confirm that formulations, containing azithromycin, can be effectively utilized for the treatment of ocular bacterial infections such as conjunctivitis, blepharitis, and keratitis. No azithromycin concentration has been detected in aqueous humor (<3.8 Tg/ml).

## CORNEA

[0045]

5	Group	Eye N.	Zone (mm)	Cs (µg/ml)	Tissue weight(g)	Ct (µg/g)
	3 instillations	1	ND		0.0421	<100
		2	ND		0.0415	<100
10		3	ND		0.0433	<100
	animals sacrificed	4	ND		0.0436	<100
	after 6 hours	5	10.00	2.62	0.0465	113
		6	ND		0.0417	<100
15		7	ND		0.0431	<100
		8	10.00	2.62	0.0482	109
	6 instillations	9	12.00	5.47	0.0406	269
		10	12.75	7.21	0.0494	292
20		11	11.25	4.15	0.0520	159
	animals sacrificed	12	10.25	2.87	0.0512	113
	after 12 hours	13	12.00	5.47	0.0483	226
		14	12.75	7.21	0.0460	313
25		15	12.25	6.00	0.0530	226
		16	11.00	3.78	0.0414	183
Average 222.5; Sd 68.7; s.e.m 24.2						
	6 instillations	17	11.75	4.99	0.0462	216
30		18	11.75	4.99	0.0586	170
		19	11.00	3.78	0.0482	157
	animals sacrificed	20	11.00	3.78	0.0522	145
	after 24 hours	21	10.50	3.14	0.0480	131
35		22	11.00	3.78	0.0550	137
		23	11.25	4.15	0.0468	177
		24	12.00	5.47	0.0495	221
Average 169.3; Sd 34.2; s.e.m. 12.1						
40	Cs: Suspension concentration of azithromycin (µg/ml)					
	Ct: Tissue concentration of azithromycin (µg/g)					
	ND: Not Detected					
	Sd: Standard deviation					
	s.e.m: standard error of the mean					

## CONJUNCTIVA

[0046]

50	Group	Eye N.	Zone (mm)	Cs (µg/ml)	Tissue weight(g)	Ct (µg/g)
	3 instillations	1	ND		0.0148	<200
		2	ND		0.0328	<200
55		3	ND		0.0342	<200
	animals sacrificed	4	11.00	4.24	0.0294	288
		5	11.00	4.24	0.0393	216



(continued)

Group	Eye N.	Zone	Cs	Tissue	Ct
		(mm)	(µg/ml)	weight(g)	(µg/g)
after 6 hours	6	10.00	3.01	0.0284	212
	7	10.00	3.01	0.0307	196
	8	10.50	3.57	0.0212	337
Average 249.8; Sd 60.3; s.e.m 26.9					
6 instillations	9	11.75	5.48	0.0084	1305
	10	13.00	8.39	0.0281	597
	11	12.25	6.50	0.0203	640
animals sacrificed after 12 hours	12	12.00	5.96	0.0258	462
	13	11.75	5.48	0.0136	806
	14	11.00	4.24	0.0191	444
	15	12.00	5.96	0.0268	445
	16	11.75	5.48	0.0262	418
Average 544.5; Sd 143.2; s.e.m 54.1					
6 instillations	17	11.25	4.62	0.0188	491
	18	11.25	4.62	0.0175	528
	19	10.75	3.89	0.0248	314
animals sacrificed after 24 hours	20	11.00	4.24	0.0228	372
	21	10.00	3.01	0.0260	232
	22	10.00	3.01	0.0115	523
	23	ND		0.0131	
	24	11.00	4.24	0.0176	482
Average 420.3; Sd 115.5; s.e.m 43.7					
Cs: Suspension concentration of azithromycin (µg/ml)					
Ct: Tissue concentration of azithromycin (µg/g)					
ND: Not Detected					
Sd: Standard deviation					

s.e.m: standard error of the mean

**EXAMPLE 5****In Vitro Antibacterial Activity**

[0047] *In vitro* bacteriostatic/bactericidal activity of azithromycin in different ophthalmic solutions has been evaluated as a function of ([H+]). Assessment of the activity it has been carried out by standardized method recommended by the National Committee for Clinical Laboratory Standards (NCCLS).

[0048] In particular, three azithromycin solutions at different pH (6.5, 7.2, 7.8) in citrate-phosphate have been examined.

[0049] Accordingly, three aliquots of Mueller-Hinton broth have been adjusted to the same pH of the azithromycin solutions (three) to be tested.

[0050] MIC values (µg/ml) have been measured by turbidity standard method after incubation of azithromycin aliquots, at the different pH, ranging from 18 to 24 h. Incubation for *Staphylococcus* spp. and *Pseudomonas* spp. needs aerobic conditions whereas *Streptococcus* spp. and *Haemophilus* spp. require partial anaerobic conditions during the period of incubation (5%, CO<sub>2</sub>).

[0051] Concentration of azithromycin able to inhibit the growth of the bacteria, after the period of incubation, is defined as the minimum inhibitory concentration (MIC).

[0052] Minimum bactericidal concentration (MBC) has been evaluated, by plating into appropriate culture medium, 0.1 ml of each azithromycin concentration above MIC. MBC of azithromycin is defined as the lowest concentration able

to determine a bacterial growth less than 10 colony forming units (cfu).

[0053] As shown in the following table, antimicrobial activity (MBC and MIC) of the three tested ophthalmic solutions are affected from pH. In particular, it has been established that is possible to select an appropriate pH range wherein the ophthalmic solutions are shown still effective to inhibit the activity of azithromycin against the most important pathogens causing ocular infections.

MIC (µg/ml)	pH			MBC (µg/ml)	pH		
	6.5	7.2	7.8		6.5	7.2	7.8
<i>S. aureus</i> ATCC 6538P	2.25	1	0.75	<i>S. aureus</i> ATCC 6538P	> 4	2.5	1.75
<i>S. aureus</i> ATCC 29213	3.25	2.25	1	<i>S. aureus</i> ATCC 29213	>4	>4	2.25
<i>S. aureus</i> ATCC 25923	3	1.5	0.75	<i>S. aureus</i> ATCC 25923	>4	3.25	2
<i>S. epidermidis</i> ATCC 12228	2.5	1.5	0.75	<i>S. epidermidis</i> ATCC 12228	>4	3	2
<i>St. Pneumoniae</i> ATCC 49619	1.25	0.25	0.15	<i>St. Pneumoniae</i> ATCC 49619	3	0.45	0.3
<i>St. Pyogenes</i> ATCC 21547	1.5	0.37	0.20	<i>St. Pyogenes</i> ATCC 21547	3	0.6	0.35
<i>H. influenzae</i> ATCC 9006	3	2	0.5	<i>H. influenzae</i> ATCC 9006	6	3.5	3.5
<i>H. influenzae</i> ATCC 49247	3	2	1	<i>H. influenzae</i> ATCC 49247	7	3.5	3
<i>Ps. aeruginosa</i> ATCC 9027	200	25	6.25	<i>Ps. aeruginosa</i> ATCC 9027	-	>200	200
<i>Ps. aeruginosa</i> ATCC 27853	>200	50	12.5	<i>Ps. aeruginosa</i> ATCC 27853	-	100	50

#### EXAMPLE 6

##### Antimicrobial activity of azithromycin eye drops

[0054] To test, reproducibly, the antimicrobial activity of azithromycin eye drops, a rabbit model of conjunctivitis has been designed. An abrasion along the inner surface of the lower lid together with a radial 4-mm incision near the medial canthus has been caused on both eyes of fifteen white New Zealand rabbits (1.8-2.0 kg). Soon after 100 TI of suspension containing  $1 \times 10^8$ /ml *S. aureus* of a clinical ocular isolate have been administered in the conjunctival *cul-de-sac* of both eyes every two hours for three times. Slit lamp examination has been performed and the signs of ocular infection monitored up to 5 day, at 24-h intervals, according to the modified McDonald-Shadduck scale. Immediately after the examination for clinical scoring, groups of two or three rabbits have been sacrificed by intravenous injection of Tanax<sup>R</sup>. At 24 h from the injury *S. aureus* produces conjunctivitis in the rabbits that remains throughout the 5-days period of ocular observation. Hyperemia and purulent discharge have been the most pronounced and persistent signs scored. This experimental model of conjunctivitis then has been applied for testing the antimicrobial activity of azithromycin eye drops (0.3%, 1%, 2%).

[0055] Fifteen male albino New Zealand rabbits, weighing 1.8-2 Kg, have been randomly distributed in groups (n=3) for being topically treated with test samples or placebo eye drops 24 h after the bacterial inoculation of *staphylococcus aureus*. The experimental design is summarized as follows:

Group	Eye	N. of eyes	Infection present	Treatment	Test substance
I	R	5	Yes	50 µl every 2h for 12h	2% eye drops
	L	5	Yes	50 µl every 2h for 12h	Vehicle only
II	R	5	Yes	50 µl every 2h for 12h	1% eye drops
	L	5	Yes	50 µl every 2h for 12h	Vehicle only

(continued)

Group	Eye	N. of eyes	Infection present	Treatment	Test substance
III	R	5	Yes	50 µl every 2h for 12h	0.3% eye drops
	L	5	Yes	50 µl every 2h for 12h	Vehicle only
R = Right L = Left					

[0056] One hour after the last administration, animals have been sacrificed by intravenous injection of Tanax<sup>R</sup>. Conjunctivae, surgically removed, have been ground in an appropriate volume of H<sub>2</sub>O 0.1% peptone with Stomacher® 80 Lab System (*pbi international*). Aliquots of the supernatant have been filtered and the residue samples have been placed on blood agar plates (Columbia CNA agar). The plates have been incubated at 37°C for 24 h, after which colony-forming units (cfu), expressed as log cfu/g of tissue have been determined for each conjunctiva.

[0057] The bacterial burden reduction in the three groups of rabbits treated with different eye drops solution of azithromycin is shown in Fig. 5. Based on this activity profile, azithromycin eye drops is considered a promising candidate for the treatment of ocular bacterial conjunctivitis.

#### EXAMPLE 7

##### Antimicrobial activity of azithromycin eye drops

[0058] A rabbit model of keratitis has been realized by application of *S. aureus* into the central corneal stroma. Both eyes of eight white New Zealand rabbits (1.8-2.0 kg) have been intrastromally injected with 10 µl of suspension containing ~1x10<sup>3</sup> ufc/ml of *Staphylococcus aureus* ocular isolate. Clinical examination by slit lamp has been performed and the correspondent signs of ocular infection monitored up to 3 day, at 24-h intervals, according to the modified McDonald-Shadduck scale. After each observation, groups of two rabbits have been sacrificed by intravenous injection of Tanax<sup>R</sup> in the marginal vein of the ear. Corneas, immediately removed after sacrifice, have been analyzed for the determination of bacterial burden. Twenty-four hours from *S. aureus* infection the loss of corneal transparency is particularly evident in the region of the inoculation. It has also been observed a conjunctival involvement with diffuse redness, and an elevated mucopurulent secretion. The clinical representation of the subsequent observations at 48-72 h makes worse with a complete loss of corneal transparency and purulent discharge. This experimental model of keratitis has been hereafter applied for testing the antimicrobial activity of azithromycin eye drops (0.3%, 1%, 2%).

[0059] Sixteen male albino New Zealand rabbits, weighing 1.8-2.0 Kg, have been randomly distributed in groups (n=3) for being topically treated with test substance or placebo eye drops 24 h after the bacterial inoculation. The experimental design is summarized as follows:

Group	Eye	N. of eyes	Infection present	Treatment	Test substance
I	R	4	Yes	50 µl every 2h for 12h	2% eye drops
	L	4	yes	50 µl every 2h for 12h	Vehicle only
II	R	6	Yes	50 µl every 2h for 12 h	1% eye drops
	L	6	yes	50 µl every 2h for 12h	Vehicle only
III	R	6	Yes	50 µl every 2 h for 12 h	0.3% eye drops

(continued)

Group	Eye	N. of eyes	Infection present	Treatment	Test substance
	L	6	yes	50 µl every 2 h for 12 h	Vehicle only
R = Right L = Left					

[0060] One hour after the last instillation, animals have been sacrificed by intravenous injection of Tanax<sup>R</sup>. Corneas, surgically removed, have been ground in 3 ml of H<sub>2</sub>O 0.1% peptone and homogenized. Aliquots of supernatant have been serially diluted (1:10) and 0.1 ml of each final suspension, including the undiluted sample, have been spread on blood agar plates (Columbia CNA agar). The plates have been incubated at 37°C for 24 h, after which colony-forming units (cfu), expressed as log cfu/g of tissue, have been determined for each cornea.

[0061] The bacterial burden reduction in the three groups of rabbits treated with different eye drops concentrations of azithromycin is shown in Fig. 6. Mean values of the bacterial burden reduction into the corneas treated with azithromycin (2%) are slightly lower than those treated with vehicle. However, in view of the fact that a favorable pharmacokinetic profile into the cornea has been determined (see EXAMPLE 4) then it is possible to believe that more elevated concentration of azithromycin (>2%), to be administered also as ointment or gel or other release systems forms, may result sufficiently effective in the ocular therapy of bacterial keratitis.

### Claims

1. A process for the preparation of an ophthalmic aqueous formulation, comprising azithromycin, which comprises the solubilization of ophthalmically acceptable polybasic phosphate in an amount ranging from 7.8 to 6.6 g/l, and citric acid monohydrate in an amount ranging from 0.9 to 35.94 g/l, and the subsequent addition of azithromycin, in an amount ranging from 0.1 to 100 g/l, within a temperature range of 15-25 °C, wherein the molar ratio of azithromycin to citric acid is equal to 1:0.67-1:1.5; wherein pH is adjusted up to a range of 5.5-7.6; by having a final osmolality equal to 130-300 mOsm/kg.
2. A process as defined in claim 1, wherein the molar ratio of azithromycin to citric acid is 1.5:1.
3. A process as defined in claim 1 or 2, wherein the ophthalmically acceptable polybasic phosphate is disodium hydrogen phosphate dodecahydrate.
4. A process as defined in any previous claims, wherein pH ranges from 6.4 to 7.6.
5. A process as defined in any previous claims which comprises, after the solubilization of azithromycin, the addition of at least a tonicity agent and/or a viscosity-increasing agent and/or a gelling agent and/or a stabilizing agent and a preservative agent, in amounts ophthalmically acceptable.
6. An aqueous ophthalmic formulation prepared in according to the process as defined in any previous claims
7. A formulation as defined in claim 6, wherein the concentration of azithromycin is equal to 0.01-10% w/v.
8. A formulation as defined in claims 6 or 7, which comprises, in combination with azithromycin, at least another, ophthalmically acceptable, therapeutic agent having antibacterial activity and/or a therapeutic steroidal or nonsteroidal agent having antiinflammatory activity.
9. A formulation as defined in claim 8, wherein the therapeutic agent having antibacterial activity is selected from the group consisting of aminoglycosides, fluoroquinolones, tetracyclines, polymyxin, glycopeptides, glycoproteins, natural and/or synthetic peptides, β-lactam derivatives.
10. A formulation as defined in claim 8, wherein the steroidal antiinflammatory agent is selected from the group made up of desonide 21-phosphate, dexamethasone, clobetasone, mometasone, betametasone and fluticasone.
11. A formulation as defined in claim 8, wherein the nonsteroidal antiinflammatory agent is selected from the group

made up of naproxen, diclofenac, nimesulide and flurbiprofen.

12. A formulation as defined in any claims indicated from 6 to 11, consisting of aqueous solution, ointment or gel.

5 13. The topical use of an ophthalmic formulation as defined in any claims indicated from 6 to 12, for the preparation of a pharmaceutical composition to be utilized in the treatment of ocular pathologies requiring antibacterial therapy.

14. The use as defined in claim 13, wherein the ocular pathology refers to conjunctivitis, keratitis or blepharitis.

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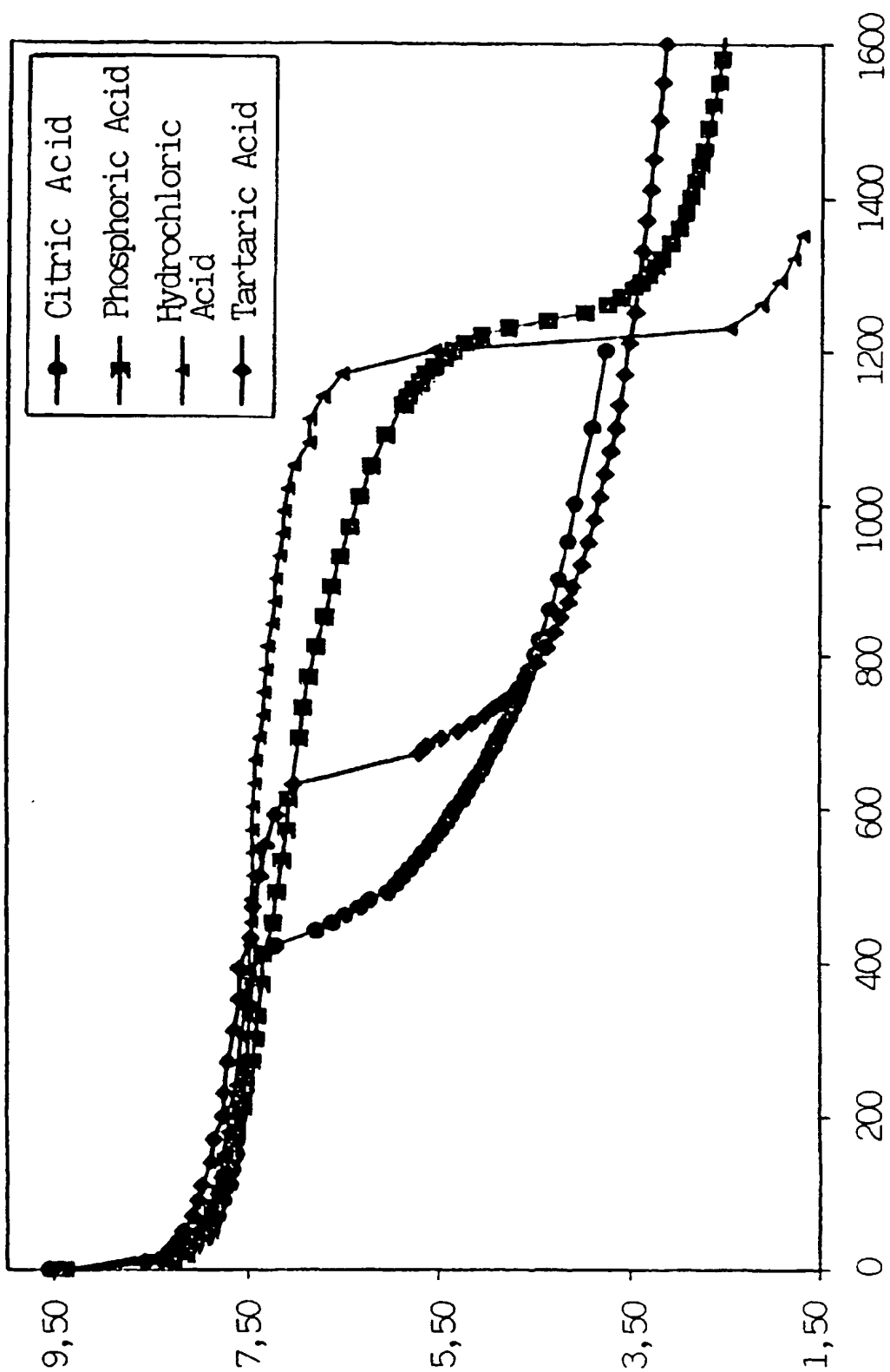


Fig. 1

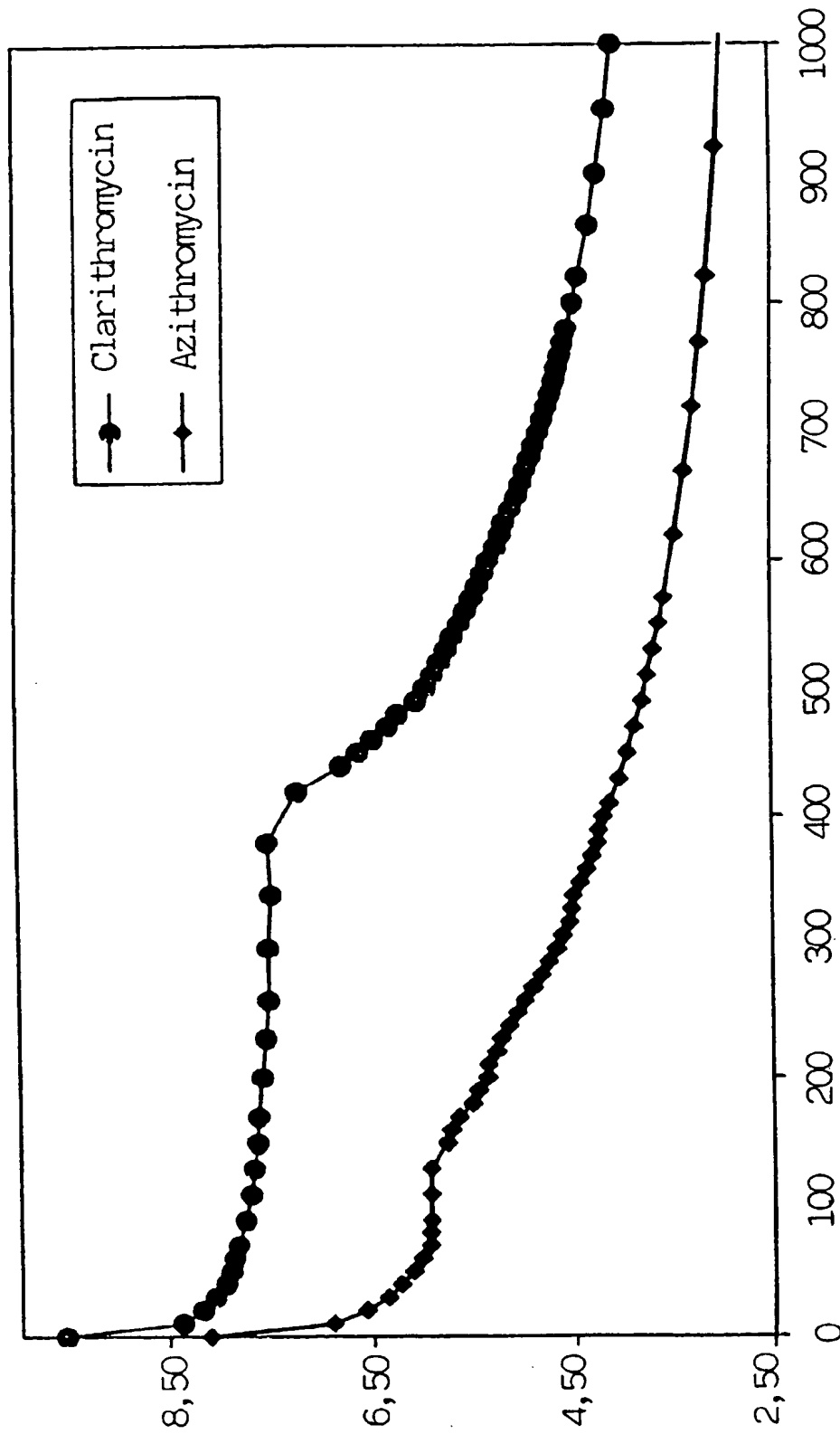


Fig. 2

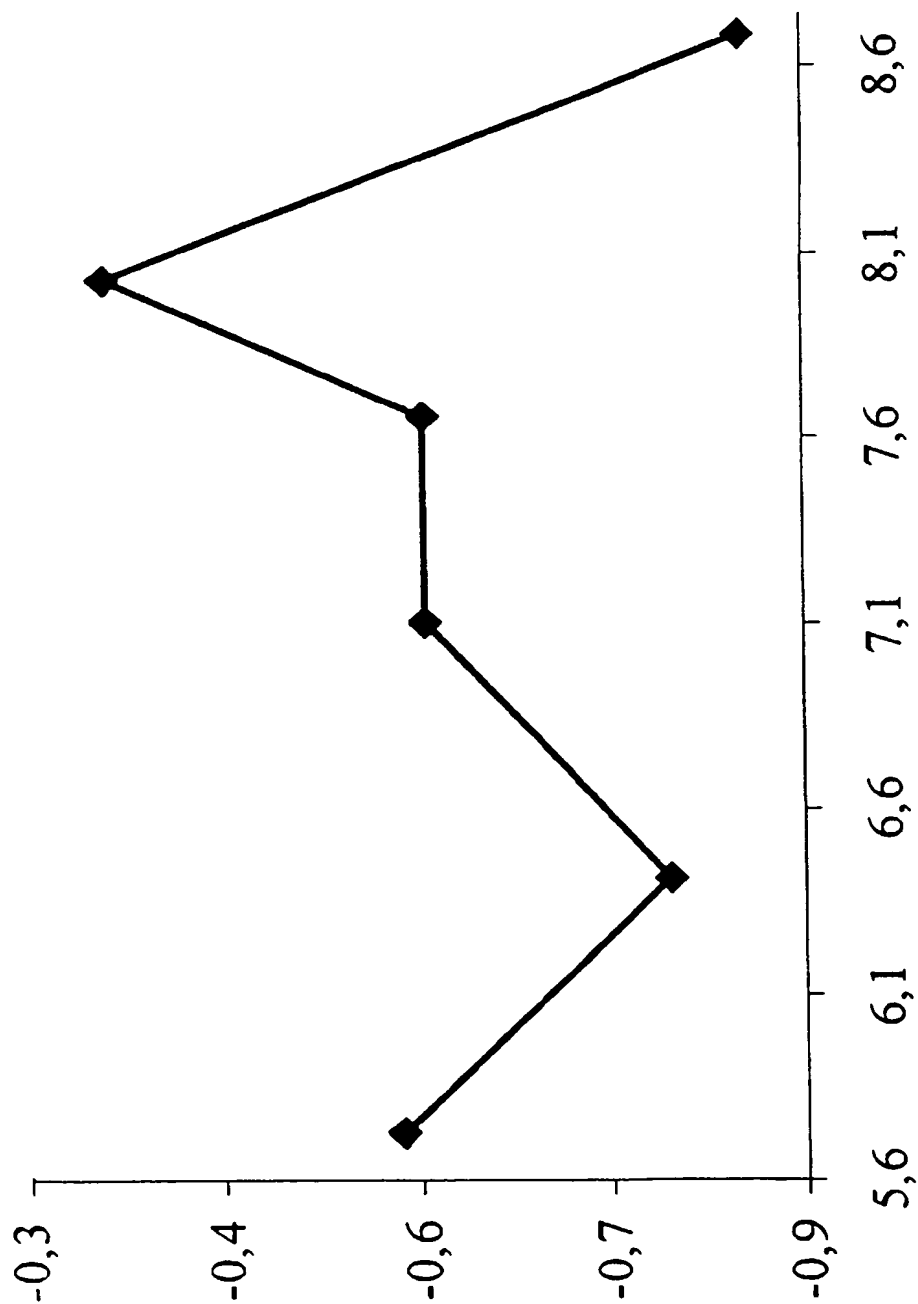


Fig. 3



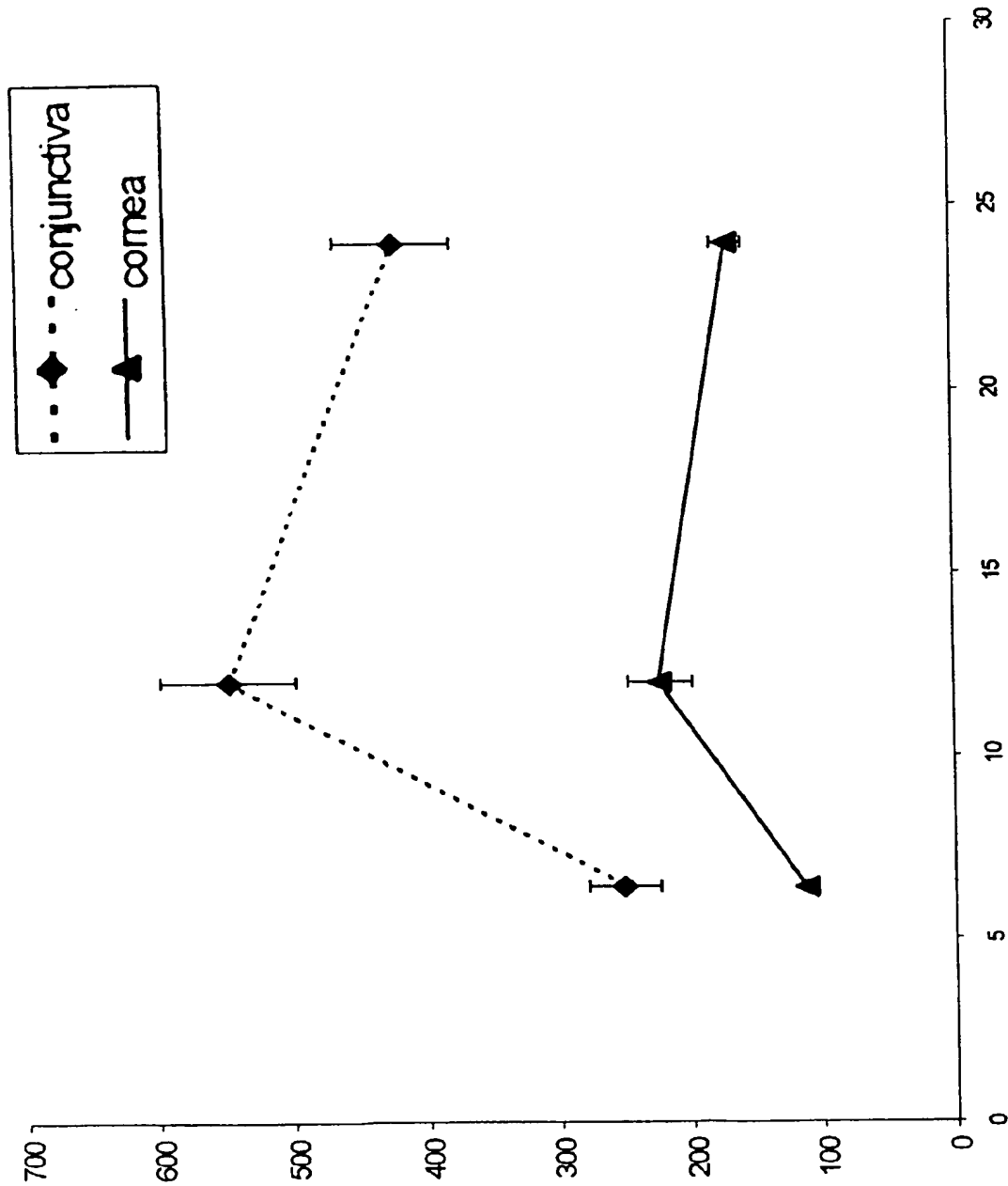
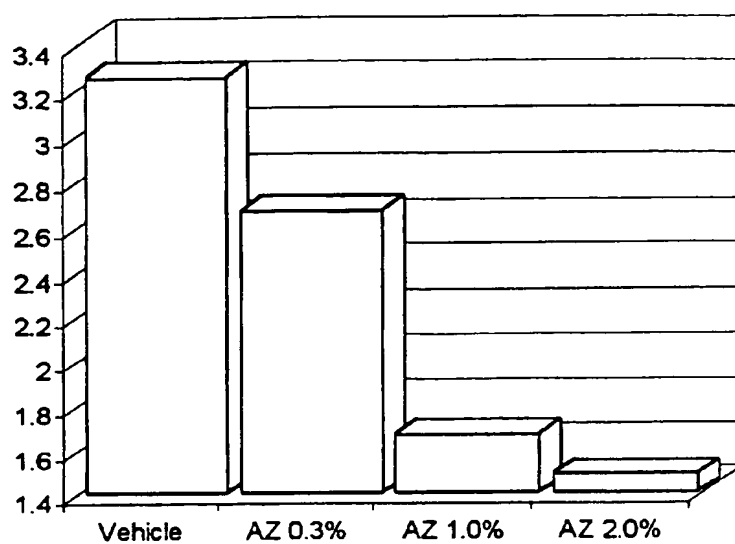
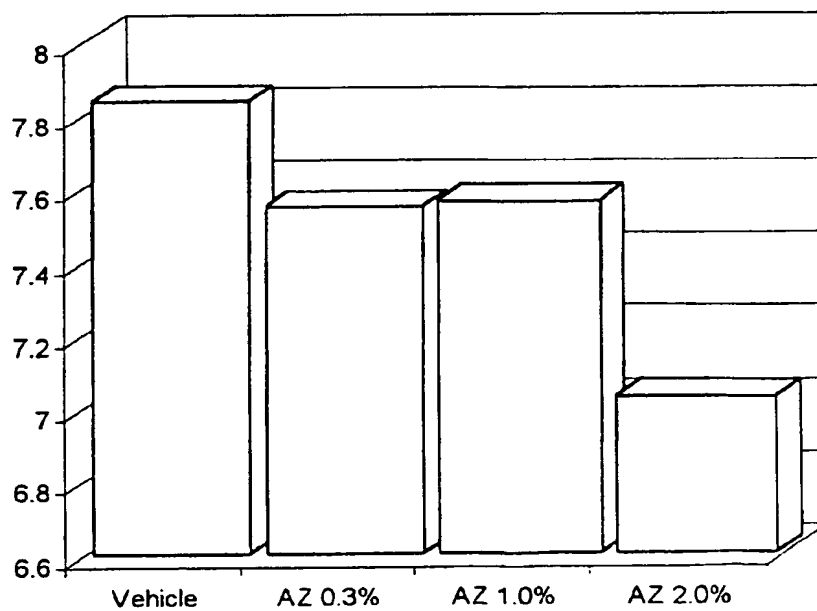


Fig. 4



*Fig. 5*



*Fig. 6*

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**(54) Process for the preparation of aqueous formulations for ophthalmic use**

(57) The present invention describes a process for the preparation of aqueous formulations containing azithromycin for ophthalmic use, as well any formulations

obtained by this process and their topical use in the ocular bacterial infections, more preferably in the treatment of conjunctivitis, keratitis and blepharitis.

**EP 1 075 837 A3**



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# PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

EP 99 12 5642

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			A61K
INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>Although claims 13 and 14 are directed to a method of treatment of the human/animal body (Article 52(4) EPC), the search has been carried out and based on the alleged effects of the composition.</p>			
Place of search		Date of completion of the search	Examiner
THE HAGUE		13 March 2001	Epskamp, S
CATEGORY OF CITED DOCUMENTS		<p>T : theory or principle underlying the invention</p> <p>E : earlier patent document, but published on, or after the filing date</p> <p>D : document cited in the application</p> <p>L : document cited for other reasons</p> <p>&amp; : member of the same patent family, corresponding document</p>	
<p>X : particularly relevant if taken alone</p> <p>Y : particularly relevant if combined with another document of the same category</p> <p>A : technological background</p> <p>O : non-written disclosure</p> <p>P : intermediate document</p>			

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